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Trans-urocanic acid, a natural epidermal constituent, inhibits human natural killer cell activity *in vitro*

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Abstract: UV irradiation has been reported to influence NK cell function both *in vitro* and *in vivo*. Since urocanic acid may mediate UV-induced immune modulation we tested the effect of *trans*- and *cis*-urocanic acid (UCA) on the cytotoxic activity of human peripheral blood lymphocytes against the erythroleukemic target cell line K562 *in vitro*. *Trans*-UCA was found to be a strong inhibitor of NK cell activity whereas *cis*-UCA had no effect. *Trans*-UCA also partially inhibited cytotoxic function of IL-2-activated NK cells and reduced IL-2-induced activation of NK cells. This is the first report describing *trans*-UCA to be active, and *cis*-UCA inactive, in regulating an immune function. In the skin, a decrease in epidermal *trans*-urocanic acid concentration by UV radiation could produce a favorable milieu for NK cell activity, and thus counteract the impairment of antigen-specific immune surveillance, induced by increased *cis*-urocanic acid concentrations.

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Introduction

Natural killer (NK) cells are capable of mediating lysis of certain malignant cells in a spontaneous non-MHC-restricted manner (1). The classical target for NK cells is the erythroleukemic cell line K562, but NK cells are also capable of lysing a variety of tumor cells, e.g. melanoma cells, and there is evidence for an *in vivo* role of NK and lymphokine-activated killer (LAK) cells in immunosurveillance of cutaneous tumors, including cutaneous malignant melanoma (2-4). Several factors are known to modulate the cytotoxic activity of NK cells. Previously, it has been reported that UV light regulates cytotoxic function of NK cells both *in vitro* (5) and *in vivo* (6). Recently, attention has been focused on the role of epidermal *trans*-urocanic acid (*trans*-UCA) and its UV-light induced photoisomer *cis*-urocanic acid (*cis*-UCA) as molecular mediator(s) of UV-mediated immune modulation (7-10). In this report we show that NK cell-mediated cytotoxicity against the erythroleukemic cell line K562 is downregulated by *trans*-UCA but not by *cis*-UCA. Furthermore, the activity of IL-2-activated killer cells is inhibited by *trans*-UCA

in high concentrations, but not by *cis*-UCA. The possible role of intracellular cAMP in inhibition of NK cell activity by *trans*-UCA is discussed.

Material and methods

Urocanic acid

Trans-UCA was purchased from Sigma Chemical Co. (St. Louis, MO). *Cis*-UCA, prepared from *trans*-UCA with UV irradiation in an alkaline solution followed by ion exchange chromatography, contained *trans*-isomer less than 2%, as detected by HPLC (11). UCA was dissolved in DMSO, diluted in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with gentamycin (50 µg/ml) and 10% fetal calf serum (FCS; Gibco, Paisley, Scotland), and sterilized through a 0.22 µm filter.

Preparation of peripheral blood lymphocytes (PBL)

Mononuclear cells were isolated from venous blood samples of healthy volunteers by Ficoll-Isoopaque (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient centrifugation. Isolated mono-

nuclear cells were depleted of monocytes by adherence to plastic tissue culture flasks (Falcon, Becton-Dickinson, Mountain View, CA) at 37°C for 1 hour in medium containing RPMI 1640 supplemented with gentamycin (50 µg/ml) and 10% fetal calf serum, hereafter referred to as complete medium. The nonadherent cells were collected and, after washes, resuspended in complete medium for use in cytotoxicity assays.

Target cells

K562, an NK-sensitive erythroleukemic cell line, was maintained in a stationary suspension culture in RPMI 1640 with 10% FCS and used as target cells in cytotoxicity assays.

Cytotoxicity assay

Cytotoxic activity was measured by a standard chromium release assay. K562 cells ($2 \times 10^6/0.2$ ml) were labeled with ^{51}Cr isotope (sodium dichromate, The Radiochemical Centre, Amersham, England) at 37°C for 1 h. After three washes with RPMI 1640, 5000 Cr-labeled cells in 100 µl of complete medium were mixed with varying densities of effector cells in 100 µl complete medium in round-bottom microtiter plates (Nunc, Roskilde, Denmark) giving effector-to-target cell ratios from 50:1 to 6:1. Each test was set up in triplicate. After 4 h incubation in humidified 5% CO_2 atmosphere at 37°C, the radioactivity released in the super-

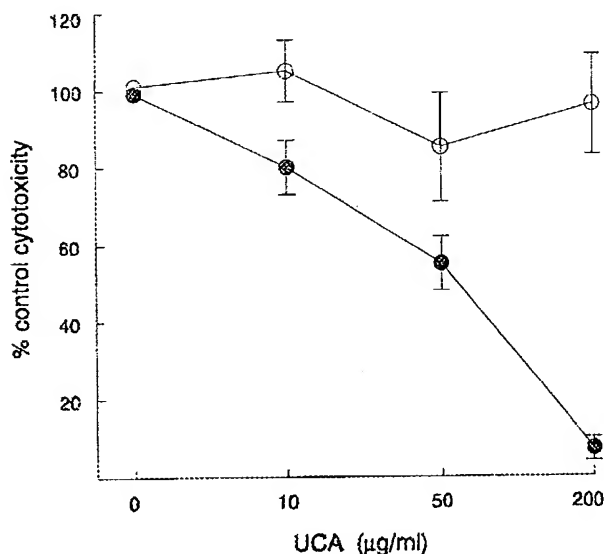


Figure 1. NK cell activity against K562 cells in the presence of urocanic acids. Cis-UCA (○) or trans-UCA (●) were added to the mixture of effector and target cells at the beginning of the 4-h incubation period. Values represent the mean \pm SD in per cent from control cytotoxicity (lysis of K562 cells in the absence of UCA) of three experiments.

Table 1. Decreased sensitivity of effector cells due to prolonged treatment with urocanic acids

UCA treatment of effector cells	Control	Cis-UCA (µg/ml)				Trans-UCA (µg/ml)			
		10	50	100	200	10	50	100	200
During 4 h assay	25 ^a	24	15	15	19	24	18	<1	<1
4 h before assay	18	19	23	19	17	25	24	19	21
20 h before assay	39	31	24	30	30	28	28	29	10

^a Percent specific lysis at an effector to target cell ratio of 25:1.

natant was counted in a well-type gamma counter (Wallac, Turku, Finland). The specific ^{51}Cr -release was determined according to the following formula: $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$.

IL-2 treatment of effector cells

PBL were resuspended at $1-2 \times 10^6$ cells/ml in complete medium and activated by incubation with 100 U/ml of recombinant interleukin-2 (IL-2; a kind gift of Dr. P. Karnani, Orion Corporation Farmos, Turku, Finland) for 20 h. At the end of the incubation period, cells were washed with RPMI 1640, resuspended in complete medium, and used as effector cells in cytotoxicity assays.

Results

In preliminary experiments, urocanic acid isomers at concentrations ranging from 10 to 200 µg/ml were added to the mixture of effector lymphocytes and K562 target cells at the beginning of a 4-h ^{51}Cr -release assay. As presented in Fig. 1, cis-UCA did not show any systematic modulation of the lytic activity of PBL. In contrast, trans-UCA at concentrations from 10 to 50 µg/ml was already slightly inhibitory, and resulted in an almost complete blocking of cytotoxicity at the concentration of 200 µg/ml. In further experiments, the effector lymphocytes were preincubated with urocanic acid isomers for 4 h and 20 h and, before addition of K562 cells, washed with complete medium (Table 1). As in preliminary experiments, concentrations of 100 to 200 µg/ml of trans-UCA very effectively blocked the cytotoxic activity of NK cells when added directly to the mixture of effector and target cells. However, the inhibition was not as marked with trans-UCA-pretreated effector cells. Preincubation with cis-UCA resulted in only a slight decrease of NK cytotoxicity against K562 cells and the effect was not dose-dependent.

The number of viable PBL was not diminished after incubation of cells with 10 and 200 µg/ml of urocanic acids when compared to PBL in control

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Table 3. Effect of *trans*-UCA and *cis*-UCA on cytotoxic activity of IL-2-stimulated peripheral blood lymphocytes^a

Urocanic acid μg/ml	IL-2	% specific lysis	
		Experiment A ^b	Experiment B ^c
none	—	35	N.D.
	+	71	69
<i>trans</i> -UCA	10	62	59
	50	82	65
	100	60	55
	200	49	26
<i>cis</i> -UCA	10	77	67
	50	71	67
	100	64	70
	200	74	69

^a PBL were incubated with 100 U/ml of IL-2 for 20 h prior to cytotoxicity assay. Cytotoxicity was measured in a 4 h ⁵¹Cr-release assay against K562 cells.

^b PBL were cultured with IL-2 in the presence of *trans*-UCA or *cis*-UCA. After stimulation, cells were washed and used as effector cells in cytotoxicity assay.

^c PBL were cultivated with IL-2 and, after washes, cytotoxicity was measured in the presence of *trans*-UCA or *cis*-UCA.

B). When added to a mixture of IL-2 activated effector cells and K562 target cells, 200 μg/ml of *trans*-UCA resulted in 62% inhibition of cytotoxicity. Again, *cis*-UCA lacked a dose-dependent inhibitory effect.

Discussion

The uppermost layer, stratum corneum, of the skin is known to contain UCA, which is synthesized in the *trans*-configuration, but upon UV irradiation is converted to its *cis*-isomer. UV irradiation has been shown to induce suppressive effects on the immune system both *in vitro* and *in vivo* (12). The original finding of DeFabo et al. was that the UV action spectrum for immunosuppression of contact hypersensitivity in mice was identical to the absorption spectrum of *trans*-UCA (7). Photoisomerization of *trans*-UCA to *cis*-UCA has been regarded as the key signal for UV-induced immunosuppression (10). Recently, this mechanism has been questioned, since contradictory evidence from photoisomerization action spectrum in mouse skin has been presented (13). In the present study, we have tested the effects of UCA isomers on human NK cell activity. A clearcut difference was found between the *cis* and *trans*-isomer, the latter strongly inhibiting native NK activity in a dose-dependent manner. Cytotoxic activity of IL-2-activated killer cells as well as their generation from PBL by IL-2 were also inhibited by *trans*-UCA, although to a lesser extent.

Previous reports, dealing with other immune

medium only, as determined by trypan blue exclusion (6.3 to 8.5×10^6 cells in *trans*-UCA-treated samples; 4.3 to 5.0×10^6 cells in *cis*-UCA-treated samples; 4.7×10^6 cells in control samples). Neither was there any difference in the relative number of NK cells after treatment of cells with 10 and 200 μg/ml of urocanic acids, as judged by monoclonal antibody staining and flow cytometry (proportion of CD16-positive cells was 10.5 to 11.1% after treatment with *trans*-UCA, 10.3 to 10.4% after treatment with *cis*-UCA, and 9.7 to 11.9% after treatment with medium only, monoclonal antibody Leu11a, and FACScan cytometer, Becton-Dickinson, Mountain View, CA).

To exclude a direct effect of urocanic acids on target cells, K562 cells were incubated with 100 μg/ml of *cis*-UCA or *trans*-UCA overnight. After washes, the cells were labeled with ⁵¹Cr and used as targets in a 4-h cytotoxicity assay. UCA-treated cells did not exhibit any increase in spontaneous lysis (data not shown). Neither *cis*-UCA nor *trans*-UCA caused any marked resistance to NK cell-mediated lysis, although treatment with *trans*-UCA resulted in slightly diminished cytotoxicity values when compared to untreated or *cis*-UCA treated K562 cells (Table 2).

To investigate the effect of urocanic acid isomers on the generation of IL-2-activated killer (LAK) cells, the effector lymphocytes were first incubated with IL-2 (100 U/ml) overnight in the absence or presence of UCA prior to testing for cytotoxicity against K562 (Table 3). A difference was observed between the effects of *cis*-UCA and *trans*-UCA on the generation of LAK cell activity (Exp. A). *Trans*-UCA inhibited the generation of LAK cell activity at the highest concentration used, although the inhibition was not complete, since the level of cytotoxicity was still about 40% higher than without IL-2 induction. The second experiment was carried out to measure the effect of urocanic acids on cells that had already been activated with IL-2 (Exp.

Table 2. Effect of urocanic acids on the susceptibility of K562 target cells to NK cell-mediated lysis

E:T ^b	Pretreatment ^a		
	Medium	<i>Trans</i> -UCA	<i>Cis</i> -UCA
50:1	52 ^c	40	53
25:1	40	33	42
12:1	27	21	28
6:1	19	15	18

^a K562 cells were incubated for 20 h with 100 μg/ml of urocanic acids. Before cytotoxicity assay, cells were washed and labelled with ⁵¹Cr. The pretreatment did not affect spontaneous lysis of K562 cells.

^b Effector to target cell ratio.

^c Percent specific lysis at a given effector to target ratio.

parameters, have implicated *cis*-UCA as the active immunomodulating isomer (10). Thus, our paper is the first to ascribe an immune regulating function to the *trans*-isomer of urocanic acid, indicating that different cell types may have different susceptibilities and responses to the two isomers of UCA. However, our results are somewhat unexpected against the background of previous data on the influence of UV radiation on NK cell function. In humans exposed to either psoralen photochemotherapy or solarium radiation, which decrease the *trans*- and increase the *cis*-isomer concentration in the skin, peripheral blood NK cell numbers and/or lytic activity have been shown to diminish (14, 15). This suppressive effect could originate in a direct exposure of circulating NK cells to UV radiation, since the solarium-induced effect was shown to be due to UVA rays (16). UVA is known to penetrate into the dermis and reach circulating blood cells (17). In fact, direct UV irradiating of human peripheral blood decreased the cytotoxicity of NK cells (18), an effect that must have occurred independently of urocanic acid which is not present in blood.

The mechanism by which *trans*-UCA inhibits NK cell activity is unknown. A direct toxic effect is unlikely, since *trans*-UCA had a transient inhibitory effect on NK cells, i.e. NK cells expressed normal levels of cytotoxicity when first pretreated with *trans*-UCA and were then, after wash-out of urocanic acid, confronted with target cells (Table 1). In addition, treatment of effector cells with *cis*-UCA or *trans*-UCA did not affect the number of viable NK cells, suggesting that *trans*-UCA mediates its effect by down-modulating the NK cytotoxic activity rather than selectively killing NK cells.

Recently, Palaszynski et al. (19) have studied the effect of urocanic acids on dermal fibroblasts and found that *trans*-UCA induces the formation of cAMP (adenosine 3',5'-cyclic monophosphate) and that *cis*-UCA reverses the effect of *trans*-UCA by preventing the synthesis of cAMP. Since increased levels of intracellular cAMP are known to suppress activation of various cell types, including cytotoxic activity of NK cells (20, 21), it is possible that a similar mechanism is operative in *trans*-UCA-induced suppression of NK cells. It has also been noted that IL-2-induced cytotoxicity is not as sensitive to cAMP-induced suppression as are native NK cells (22). Analogous findings were obtained in the present study with *trans*-UCA. We are currently investigating the role of cAMP in the *trans*-UCA-induced suppression of NK cell function.

It has been postulated that the antigen-specific tolerance of T cells that results from an altered antigen presentation capacity in UV-irradiated, *cis*-

UCA-enriched skin, has evolved to prevent autoimmune reactivity towards UV-injured cutaneous tissue (10). As a drawback of such an immunosuppressive mechanism, tolerance may be generated to cutaneous neoplastic antigens (23). Our data indicate that the antigen non-specific NK cell-mediated immune surveillance mechanism may be regulated in the opposite way by UV irradiation. In non-irradiated epidermis the prevalence of *trans*-UCA may function to keep NK cell activity under control. In UV-irradiated epidermis, where *trans*-UCA levels are diminished, NK cell activity may be favored. NK cells could thus counteract the UV light-induced impairment of the antigen-specific immune surveillance.

Acknowledgments

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